

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 441 763 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
21.05.1997 Bulletin 1997/21

(51) Int Cl.⁶: **C12N 15/16, C12P 21/02,
C07K 14/475**

(21) Application number: **91870003.0**

(22) Date of filing: **07.01.1991**

(54) Heparin-binding growth factor

Heparin bindender Wachstumsfaktor

Facteur de croissance liant l'héparine

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(30) Priority: **08.01.1990 US 462156
21.06.1990 US 542232**

(43) Date of publication of application:
14.08.1991 Bulletin 1991/33

(73) Proprietor: **The Jewish Hospital of St. Louis
St. Louis, Missouri (US)**

(72) Inventors:
• **Deuel, Thomas Franklin
Clayton, MO 63105 (US)**
• **Li, Yue-Shen
Bridgeton, MO 63044 (US)**
• **Milner, Peter Gerard
Clayton, MO 63105 (US)**

(74) Representative: **Allard, Susan Joyce et al
BOULT, WADE & TENNANT
27 Furnival Street
London EC4A 1PQ (GB)**

(56) References cited:
EP-A- 0 326 075

- **BIOCHEMICAL & BIOPHYSICAL RESEARCH COMMUNICATIONS**, vol. 165, no. 3, 29 December 1989, Academic Press Inc.; P.G. MILNER et al., pp. 1096-1103
- **THE EMBO JOURNAL**, vol. 8, no. 10, October 1989; H. RAUVALA, pp. 2933-2941
- **JOURNAL OF BIOLOGICAL CHEMISTRY**, vol. 265, no. 31, 05 November 1990, Baltimore, MD (US); M.D. KUO et al., pp. 18749-18752
- **BIOCHEMICAL & BIOPHYSICAL RESEARCH COMMUNICATIONS**, vol. 172, no. 2, 30 October 1990, Academic Press Inc., Duluth, MN (US); I. KOVESDI et al., pp. 850-854
- **SCIENCE**, vol. 250, 1990; Y.-S. LI et al., pp. 1690-1694
- **BIOCHEMICAL & BIOPHYSICAL RESEARCH COMMUNICATIONS**, vol. 173, no. 1, 30 November 1990; K.-I. TEZUKA et al., pp. 246-251

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

This invention relates to a novel peptide growth factor and, more particularly, to a heparin-binding growth factor derived from human placenta.

In recent years a considerable number of growth factors derived from various animal cells have been isolated and characterized. Illustrative of these growth factors are nerve growth factor (NGF) which has been purified from several different cell sources, insulin-like growth factors (IGF-I and IGF-11), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), endothelial cell growth factor (ECGF), somatomedins and transforming growth factors (TGF) derived from various tumors and virally transformed cells. For background information on these growth factors see, for example, the recent brief review articles by Kris et al., *Biotechnology* 3, 135-140 (1985); Dijk and Iwata, *Ibid.* 7, 793-798 (1989); and the comprehensive review in *Hormonal Proteins and peptides*, Ed. by Choh Hao Li, Vol. 12, "Growth Factors," Academic Press, 1984.

Although the existence of acidic and basic fibroblast growth factor (aFGF and bFGF) has been known for fifty years [Trowell, et al., *J. Exp. Biol.* 16, 60-70, (1939); Hoffman, *Growth* 4, 361-376 (1940)], only recently have they been purified and sequenced [Thomas et al., *Proc. Natl. Acad. Sci. USA* 81, 357-361 (1984); Gimenez-Gallego et al., *Science* 230, 1385-1388 (1985); Lemmon and Bradshaw, *J. Cell. Biochem.* 21, 195-208 (1983); Bohlen et al., *Proc. Natl. Acad. Sci. USA* 81, 5364-5368 (1984); and Esch et al., *Proc. Natl. Acad. Sci. USA* 82, 6507-6511 (1985)]. aFGF and bFGF have 55% sequence homology, suggesting that they arose by duplication and divergence from a common ancestral gene. Cells responding to the FGFs have between 10^3 and 10^5 high affinity receptors per cell [Neufeld and Gospodarowicz, *J. Biol. Chem.* 261, 5631-5637 (1986)]. The bFGF receptor has recently been purified, sequenced, and cloned [Lee et al., *Science* 245, 57-60 (1989)]. Both bFGF and aFGF compete for the same receptor and displace each other in radio-receptor assay [Neufeld, *supra*; Olwin and Hauschka, *Biochemistry* 25, 3487-3492 (1986)]; however, two forms of the receptor appear to have different affinities for aFGF and bFGF. Recently five additional proteins have been reported by cDNA cloning to have homology to the FGFs (hst, int-2, FGF-5, FGF-6, and KGF or FGF-7) [Dickson and Peters, *Nature* 326, 833 (1987); Yoshida et al., *Proc. Natl. Acad. Sci. USA* 84, 7305-7309 (1987); Delli Bovi et al., *Cell* 50, 729-737 (1987); Zhan et al., *Mol. Cell. Biol.* 8, 3487-3495 (1988); Marcis et al., *Oncogene* 4, 335-340 (1989); and Finch et al., *Science* 245, 752-755 (1989)]. All five cDNA sequences encode signal peptides, therefore suggesting that these five proteins are presumably secreted, unlike aFGF or bFGF which lack signal peptides.

In accordance with the present invention a novel heparin-binding growth factor has been isolated from human placenta. This novel heparin-binding growth factor, herein also designated as HBGF-8, is a 18.9 kDa polypeptide with a unique 25 N-terminal amino acid sequence as follows:

HBGF-8

Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-

Cys-Gly-Glu-Trp-Gln-Trp-Ser-Val-Cys-Val-Pro.

This novel growth factor binds tightly to cation exchange resins and to Heparin-Sepharose® and is stable to acetone precipitation and labile in acid.

HBGF-8 was as active as acidic fibroblast growth factor (aFGF) and slightly less active than bFGF in the mouse NIH 3T3 fibroblast mitogenic assay system with an intrinsic specific activity of 5000 dpm/ng under standard assay conditions.

In an illustrative example and based upon total activity in the acetone extracts of bovine uterus stimulating ^3H -thymidine incorporation into DNA of serum-starved NIH 3T3 cells, a 6940 fold purification was achieved with an overall yield of HBGF-8 activity of 0.4%, using extraction of acetone powders and chromatographic separations at neutral pH. Approximately 18 μg protein was obtained from 1.2 kg wet weight of tissue. HBGF-8 was clearly separated from 17.5 kDa bovine uterus basic fibroblast growth factor (bFGF) by purification and its N-terminal amino acid sequence analysed. The unique sequence as set forth above was found.

In accordance with another aspect of the invention, the complete coding sequence of cDNA clones representing the full size human HBGF-8 have been developed.

The human HBGF-8 cDNA sequence was isolated from human placenta cDNA library using bovine HBGF-8 cDNA fragment as a probe. The bovine HBGF-8 cDNA sequence was isolated from bovine uterus cDNA library using the above HBGF-8 N-terminal sequences as a probe. The cDNA sequence contains 995 nucleotides and encodes a 18.9 kDa ($M_r = 18,942$) protein of 168 amino acids, including a 32 amino acid lead sequence. The cDNA coding sequence

starts at nucleotide position 252 to 755.

Comparison of the bovine and human HBGF-8 showed that HBGF-8 is highly conserved. Of 168 amino acids, 163 were identical. Differences occur at amino acid positions 3, 4 and 7 in the leader sequence and at amino acid positions 130 and 147 in the mature protein of the complete 168 amino acid sequence.

The human and bovine cDNA protein sequences and the human and bovine cDNA sequences for HBGF-8 are as follows:

Human HBGF-8 Protein

[illegible]

Bovine HBGF-8 Protein

5
 Met Gln Thr Pro Gln Tyr Leu Gln Gln Arg Arg Lys Phe Ala Ala 15
 10 Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala 30
 Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser 45
 Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly 60
 15 Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu 75
 Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn 90
 Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala 105
 20 Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly 120
 Ser Leu Lys Arg Ala Leu His Asn Ala Asp Cys Gln Lys Thr Val 135
 25 Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Ser Lys Pro Gln 150
 Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys 165
 Met Leu Asp 168

30

35

40

45

50

55

HUMAN HBGF-8 cDNA

5	GTCAAAGGCA GGATCAGGTT CCCC GCCTTC CAGTCCAAAA ATCCCGCCAA	50
	GAGAGCCCCA GAGCAGAGGA AAATCCAAAG TGGAGAGAGG GGAAGAAAGA	100
10	GACCAGTGAG TCATCCGTCC AGAAGGCGGG GAGAGCAGCA GCGGCCCAAG	150
	CAGGAGCTGC AGCGAGCCGG GTACCTGGAC TCAGCGGTAG CAACCTCGCC	200
	CCTTGCAACA AAGGCAGACT GAGCGCCAGA GAGGACGTTT CCAACTCAA	250
15	AATGCAGGCT CAACAGTACC AGCAGCAGCG TCGAAAATTT GCAGCTGCCT	300
	TCTTGGCATT CATTTTCATA CTGGCAGCTG TGGATACTGC TGAAGCAGGG	350
	AAGAAAGAGA AACCAGAAAA AAAAGTGAAG AAGTCTGACT GTGGAGAATG	400
20	GCAGTGGAGT GTGTGTGTGC CCACCACTGG AGACTGTGGG CTGGGCACAC	450
	GGGAGGGCAC TCGGACTGGA GCTGAGTCCA AGCAAACCAT GAAGACCCAG	500
25	AGATGTAAGA TCCCCTGCAA CTGGAAGAAG CAATTTGGCG CGGAGTGCAA	550
	ATACCAGTTC CAGGCCTGGG GAGAATGTGA CCTGAACACA GCCCTGAAGA	600
	CCAGAACTGG AAGTCTGAAG CGAGCCCTGC ACAATGCCGA ATGCCAGAAG	650
30	ACTGTCACCA CTCCCAAGCC CTGTGGCAAA CTGACCAAGC CCAAACCTCA	700
	AGCAGAATCT AAGAAGAAGA AAAAGGAAGG CAAGAAACAG GAGAAGATGC	750
	TGGATTAAAA GATGTCACCT GTGGAACATA AAAAGGACAT CAGCAAACAG	800
35	GATCAGTTAA CTATTGCATT TATATGTACC GTAGGCTTTG TATTCAAAAA	850
	TTATCTATAG CTAAGTACAC AATAAGCAAA AACAAAAAGA AAAAAAAAAA	900
40	AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	950
	AAAAAAAAAA A	961

45

50

55

Bovine HBGF-8 cDNA

5	GAGTGGAGAG AGTAGAAGAA AGAGAGCAGG GAGTCACCGG GCGTGCAGGG	50
	GCGGAGAGCA GCGGCCGCCG CGAGCACCAG CGACTTGGGT ACCTGGACTC	100
10	AGGGCAGAAA AACCTCTCCC GGATCAACAA AGGCAGCCTG AGCGCGCACC	150
	GCTCTTTTGC GACTCCAAAA TGCAGACTCC ACAGTACCTG CAGCAACGTC	200
	GAAAATTTGC AGCTGCCTTT TTGGCATTTA TTTTCATCTT GGCAGCTGTG	250
15	GACACCGCTG AAGCAGGAAA GAAAGAGAAA CCAGAAAAGA AGGTGAAGAA	300
	GTCTGACTGT GGAGAAATGGC AGTGGAGTGT GTGTGTACCA ACCAGTGGGG	350
	ACTGTGGGCT GGGCACCCGC GAGGGCACCC GTACCGGAGC TGAGTGTAAG	400
20	CAAACCATGA AGACCCAGAG ATGTAAGATC CCCTGCAACT GGAAAAAGCA	450
	ATTTGGAGCG GAGTGCAAAT ACCAGTTCCA GGCCTGGGGA GAATGTGATC	500
25	TGAACACGGC TCTGAAGACC CGAACTGGGA GCCTGAAGCG AGCCCTCCAC	550
	AACGCCGACT GCCAGAAGAC AGTCACCATC TCCAAGCCCT GTGGCAAGCT	600
	GACCAAGTCC AAACCTCAAG CAGAATCTAA GAAGAAGAAA AAGGAAGGCA	650
30	AGAAACAGGA GAAGATGCTG GACTAAAAGC CACCACCTTC CGTGGACCAT	700
	GAAAAGGACA TCAGCAAACA CGATCAGTTA ACTATTGCAT TTATATCTAC	750
	CGTAGGCTTT TTATTCAAAA ATTATCTATA GCTTAAGTAC ACAATAGGCA	800
35	GAAACAAAAT GAAAAGAAAA ATTTTGTAGT AGCATTTTTTT TTAAATGTAT	850
	CAATATACCA TAGTACCACT AGGGACTTAT AATAGAGGAC TGTAATCCTA	900
40	TTTAGAATGT TGAATTATAG TACATGTTAA GTGATAGAAA ACTGAGGTAA	950
	GTTTTTTGAA GTTATGTGAT ATTTTACATT ACATTTTTTTT TTACATTTTTT	1000
	TTCTTTTGGC AGCAATTAA ATGTTATGAC TATGTAACT ACTTCTCTTG	1050
45	TTAGGTAATT TTTTTCACCT AGACTTTATT TCCCAATTGA GAAAAATATC	1100
	TACTAAACAA AGCAGCAATA AAATATGATC ATCCTATCTG AGGAAAATAT	1150
50	CTCTTTTTCT GCCAGTGGAT TTTTAAAAA TTGTAGTCAA CAAAAT	1196

In the bovine HBGF-8 protein sequence, the following amino acid replacements of the human sequence are found:

55 Ala-3-Thr; Gln-4-Pro; Gln-7-Leu; Glu-130-Asp;
and Pro-147-Ser.

Although the present invention is directed to a heparin-binding growth factor derived from human placenta, it is considered that assistance in understanding the invention will be obtained from the following detailed description of heparin-binding growth factor derived from bovine uterus taken in connection with the accompanying drawings in which briefly:

FIG. 1 is a graphical representation which shows Chromatographic Separation of Bovine Uterus Mitogenic Activity. The mitogenic activity that bound to Heparin-Sepharose between 900 mM and 2000 mM NaCl was concentrated from 1160 ml to 12 ml using a MonoS HR 10/10 column, elution of this column using 2000 mM NaCl, and reapplication following dilution to 500 mM NaCl to the MonoS HR 5/5 column shown above. The A_{280} absorption profile elution of protein is shown by the shaded area. The results of the solid phase ELISA to detect bovine bFGF immunoreactivity are shown by the open triangles. In addition, the mitogenic activity in the mouse NIH 3T3 assay system is shown by the open squares. The NaCl gradient that was applied from 500 mM to 2000 mM salt is shown by the dashed line and individual fractions of 1 ml were collected. The bFGF immunoreactivity is expressed as $\mu\text{g/ml}$ of bFGF. The region of the column shown by the continuous line and labeled region A was pooled, dialyzed, concentrated and applied to the microbore cation exchange column.

FIG. 2 is a graphical representation which shows cation exchange chromatographic separation of mitogenic proteins from bovine uterus. Region A (Fig. 1) was applied to a 2.1 x 220 nm Aquapore CX-300 cation exchanger and a 0-2 M NaCl gradient applied. The gradient is shown by the dashed line and the elution pattern of the proteins or A_{280} by the continuous line; the histogram shows the mitogenic activity of the fractions in the NIH 3T3 assay system. The HBGF-8 peak of mitogenic activity can be seen to elute at molarity of 1-1.2 M salt.

FIG. 3 shows the SDS-PAGE of the pure bovine uterus bFGF and HBGF-8. Lane 1 shows the appearance after SDS-PAGE in 15% gels of the pure 17.5 kD bovine uterus bFGF following reverse phase C4 chromatographic purification. This material was used for N-terminal sequencing. Lane 2 shows the appearance of the 17 kD novel HBGF-8 in the same gel system. This tube was used for determining novel N-terminal amino acid sequence shown above. The molecular weight markers used were BRL low molecular weight range markers.

In order to illustrate specific preferred embodiments of the invention in greater detail, the following exemplary laboratory preparative work was carried out. It will be appreciated, however, that the invention is not limited to these specific examples or the details described therein.

EXAMPLE 1

METHODS

Mitogenic Assays. The mitogenic activity of samples was assayed in triplicate by measuring the incorporation of [methyl- ^3H] thymidine into acid-insoluble DNA of confluent, serum-starved NIH 3T3 cells. Unstimulated cells were used as background and values subtracted as background, which averaged 1500 dpm.

Immunological Methods. Rabbit anti-bFGF polyclonal antisera were obtained from Dr. Judith Abraham (California Biotechnology, Inc.). The solid phase immunosorbant assay used to screen column fractions was carried out by conventional procedure as previously described by Yeh et al., *Proc. Natl. Acad. Sci. USA* 84, 2317-2321 (1987).

Extraction of Growth Factor Activity from Acetone Powders. The acetone powders were prepared by conventional procedure as previously described by Porter et al., *J. Biol. Chem.* 263, 8989-8995 (1988), extracted with 40 ml/g of 0.9 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM [ethylenebis(oxyethylenenitrilo)] tetra-acetic acid (EGTA), 0.15 mg/ml phenylmethylsulphonyl fluoride, 10 mM benzamidine, 0.1 mg/ml soybean trypsin inhibitor, and 1 $\mu\text{g/ml}$ aprotinin for 2 h at 4°C with agitation, and centrifuged at 10,000 x g for 30 min.

Heparin-Sepharose Chromatography. 6.9 liters of the soluble extract (34.5 g protein) was diluted to 20.7 liters in 900 mM NaCl, 20 mM Hepes, pH 7.0, loaded onto a 5 x 12.5 cm Heparin-Sepharose (Pharmacia) column at 4°C at a flow rate of 5 ml/min, washed in 10 column volumes of the starting buffer, 20 mM Hepes, pH 7.0, 900 mM NaCl, and eluted with 1160 ml of 20 mM Hepes, pH 7.0, 2 M NaCl, at a flow rate of 1 ml/min. The eluted fraction (2 M NaCl) was dialyzed against 20 mM MES, pH 6.0. Hepes = N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]. MES = 2[N-Morpholino]ethanesulfonic acid.

MonoS Chromatographic Separation of Mitogenic Activity. The active fraction (20 mM MES, pH 6.0) was loaded onto a MonoS HR 10/10 column (Pharmacia, FPLC) at 2.5 ml/min, washed with 10 column volumes of starting buffer, eluted with 12 ml (1.5 column volumes) of 20 mM MES, pH 6.0, 2 M NaCl, at a flow rate of 2.5 ml/min., diluted 1:4 with 20 mM MeS, pH 6.0 (final volume, 48 ml, 20 mM MES, pH 6.0, 500 mM NaCl), and loaded at 0.5 ml/min onto a MonoS HR 5/5 column (Pharmacia). Following loading, the column was washed with 10 column volumes of loading buffer and eluted with a 33 ml gradient from 500 mM NaCl to 2 M NaCl in 20 mM MES (pH 6.0). The column was further eluted with 2 M NaCl (10 ml) and the 1 ml fractions assayed in the NIH 3T3 mitogenic assay system. Each fraction also was assayed for bFGF immunoreactive proteins using a solid phase ELISA.

Microbore Cation Exchange Chromatography. The fractions (region A, Fig. 1, Mono HR 5/5 column) were pooled,

dialyzed against 2 mM Hepes, pH 7.0, concentrated (1 ml), and loaded on a 2.1 x 220 mm aquapore CX-300 weak cation exchange column (Brownlee Labs) at a flow rate of 200 µl/min (Applied Biosystems, Inc., Model 130A Microbore HPLC system). The column was eluted with a 0-2 M NaCl gradient (15 ml, 20 mM Hepes, pH 7.0) at a flow rate of 200 µl/min and fractions assayed in the NIH 3T3 assay system for mitogenic activity.

Reverse Phase Chromatographic Purification of bFGF. The fractions containing immunoreactive bFGF were loaded on a 2.1 x 220 nm Aquapore BU-300 C4 reverse phase column (Brownlee Labs, Applied Biosystems HPLC System), the column equilibrated with 100% water, 0.1% TFA, and eluted with a 15 ml gradient from 0-100% acetonitrile/0.1% TFA at a flow rate of 200 µl/min. TFA = trifluoroacetic acid.

N-Terminal Amino Acid Sequencing. Fractions were analyzed by repetitive sequence analysis on a gas phase sequencer (Model 470A, Applied Biosystems, Inc.) essentially as described by Hunkapiller et al., *Meth. Enzymol.* 91, 399-412 (1983). The respective PTH-aa derivatives were identified using an Applied Biosystems Model 120A PTH analyzer. The initial yields on sequencing both proteins were 185 and 2475 pmoles for the bFGF and HBGF-8, respectively. Recoveries averaged greater than 95% for both sequences.

RESULTS

Purification of Heparin-Binding Growth Factor-B (HBGF-8). The purification scheme for bovine uterine HBGF-8 is shown in Table 1. 1.2 kg of frozen bovine uterus yielded 18 µg of nearly pure HBGF-8 with an intrinsic specific activity in an NIH 3T3 cell mitogen assay of 5000 dpm/ng. Comparable values for aFGF and bFGF were 4090 and 9000 dpm/ng, respectively. Ten µg bFGF per 1.2 kg wet weight of bovine uterus was also obtained (see alternative method, above). An overall recovery of 0.4% of HBGF-B activity with a 6944 fold purification was obtained; because uterus contains other growth factors active in mitogenic assays using NIH 3T3 cells as targets, the recovery is grossly underestimated due to the initial inclusion of all growth factors active against NIH 3T3 cells in assay of crude extracts of bovine uterus.

The MonoS HR 5/5 column used to further separate the mitogenic proteins binding to Heparin-Sepharose is shown in Fig. 1. bFGF immunoreactivity and mitogenic activity are shown. bFGF eluted at 0.8 M NaCl; it is both immunoreactive and a potent mitogen in the NIH 3T3 assay system. Importantly, significant activity bound to MonoS more tightly than bFGF; this data first suggested the presence of a novel growth factor.

The fractions indicated by the solid bar, region A, were pooled, dialyzed, concentrated and loaded onto the microbore CX-300 cation exchange column for elution with a 0.0-2.0 M NaCl gradient. The mitogenic activity identified (Fig. 2) corresponded to a 17 kD protein when analyzed in SDS-PAGE gels under reducing conditions. The appearance of the 17.5 kD bFGF and the novel 17 kD HDGF-8 by SDS-PAGE are shown in Fig. 3.

N-Terminal Amino Acid Sequencing. The separation of bFGF from HBGF-8 was pursued by N-terminal amino acid sequencing. The bFGF was further purified by C4 reverse phase chromatography to yield a single silver-stainable band at 17 kD on SDS-PAGE. N-terminal amino acid sequencing revealed N-terminal sequences for the bovine uterus bFGF proteins as shown in Table II. The N-terminal sequence confirmed that there is an N-terminal 2 amino acid extension beyond the first methionine sequenced (at position 3) which, during initial purifications of bFGF, was thought to be the site of initiation [Abraham et al., *EMBO J.* 5, 2523-2528 (1986)]. This N-terminal sequence obtained corresponded precisely with the N-terminal amino acid sequence for human placental bFGF [Sommer et al., *Biochem. Biophys. Res. Commun.* 144, 543-550 (1987)]. Alanine-2 in the bovine uterus bFGF sequence is substituted for threonine-2 in the human bFGF sequence.

A novel 25 amino acid N-terminal sequence was then obtained for HBGF-8, as shown in Table II, confirming the separation of two high affinity heparin-binding growth factors from bovine uterus. The sequence is extremely rich in lysines (7 of the first 12 amino acids). A search of both NBRF and GENBANK data bases failed to reveal sequence homology to any known protein or gene product. Thus, although bFGF and HBGF-8 elute nearly together from various columns and migrate nearly identically by SDS-PAGE, each may be separated from the other and each have different N-terminal amino acid sequence.

HBGF-8 is both a potent mitogen and abundant in uterus. It has an intrinsic specific activity in the NIH 3T3 assay system which is roughly equal within methods of assay to aFGF and bFGF. aFGF could not be detected among the growth factors in bovine uterus which bound to Heparin-Sepharose at 900 mM NaCl. The 25 amino acid N-terminal sequence of HBGF-8 has not been identified in any of the seven members of the FGF family; aFGF [Gimenez-Gallego, *supra.*], bFGF [Sommer et al., *supra.*], int-2 [Dickson and Peters, *supra.*], hst [Yoshida et al., *supra.*], FGF-5 [Zhan et al., *supra.*], FGF-5 [Marcis et al., *supra.*], FGF-6 [Finch et al., *supra.*].

HBGF-8 was purified with aqueous columns with pH values between 6.0 and 7.0. About 18 µg/kg wet weight of tissue of HBGF-8 and ~ 10 µg/kg wet weight tissue of bFGF were recovered. However, it is likely that there may be more HBGF-8 in uterus than 18 µg/kg if all the activity which binds more tightly than bFGF in Fig. 1 is due to HBGF-8. Recently, aFGF has been shown to be more abundant than formerly thought with a tissue level of 500 µg/kg in heart [Sasaki et al., *J. Biol. Chem.* 264, 17606-17612 (1989)].

TABLE I. HBCF-8 purification scheme

	Total protein (mg)	Volume (ml)	Mitogenic activity* (dpmx10 ⁶)	Specific activity§	Degree purification	Yield (%)
Solubilize acetone extract	†34,500	6,900	25,000	0.72	0	100
900-2000mM elution off heparin-sepharose	23	1,160	2,900	126	175	11.6
0-2000mM elution off MonoS HR 10/10	3	12	433	144	200	1.7
500-1000mM elution off MonoS HR 5/5	0.2	10	250	1250	1736	1.0
0-2000mM elution off CX-300 cation exchange column	0.018	0.6	90	5000	6944	0.4

*NIH 3T3 cell assay

§Units of specific activity are dpmx10⁶/mg protein

†1.2Kg frozen bovine uterus yields 207g acetone insoluble powder, which on dissolving in 900M NaCl, 50mM Hepes pH 7.0, produced solution containing 34.5g of protein.

TABLE II. Bovine uterus bFGF and HBGF-8 N-terminal sequence

<u>bFGF</u>	
80%	GLY-ALA-MET-ALA-ALA-GLY-SER-ILE-THR-THR-LEU-PRO-ALA-LEU-
10%	MET-ALA-ALA-GLY-SER-ILE-THR-THR-LEU-PRO-ALA-LEU-
10%	PRO-ALA-LEU-
<u>HBGF-8</u>	
95%	GLY-LYS-LYS-GLU-LYS-PRO-GLU-LYS-LYS-VAL-LYS-LYS-SER-ASP-
	CYS-GLY-GLU-TRP-GLN-TRP-SER-VAL-CYS-VAL-PRO.

EXAMPLE 2

Male term placenta was flash-frozen as 1 cm dice in liquid nitrogen and stored at -70°C. Total cellular and poly(A) + RNA was prepared from placenta according to the conventional procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979), as modified by Sadler et al., *Proc. Natl. Acad. Sci. USA* 82, 6394-6398 (1985). cDNA was synthesized and constructed in λ gt11 arms and packaged by the published method of Ye et al., *J. Biol. Chem.* 262, 3710-3725 (1987). Approximately 1×10^6 clones from the human placenta cDNA library were screened with a bovine cDNA probe. The screening procedure was as follows: the recombinant phages were replicated onto nitrocellulose filters and hybridized overnight at 42°C with random primer-labeled bovine cDNA probe using [α - 32 P] dCTP (3,000 Ci/mmol, NEN), in 50% formamide, 50 mM sodium phosphate (pH 7.0), 5x Denhardt's Solution, 0.1 mg/ml purified tRNA and 5x standard saline citrate (SSC) (1x SSC is 150 mM sodium chloride, 15 mM sodium citrate). Filters were washed with 0.2x SSC and 0.1% SDS at 68°C and exposed to Kodak X-Omat AR film. After tertiary screening, a positive cDNA clone was subcloned into Bluescript (Stratagene). The nucleotide sequences were obtained by the well-known dideoxy chain termination method of Sanger et al., *Proc. Natl. Acad. Sci. USA* 74, 5463 (1977), with Sequenase version 2.0 (U.S. Biochemicals). The sequences were obtained by sequencing both strands of DNA twice with different primers.

The human HBGF-8 cDNA sequence revealed an open reading frame which encodes a putative 168 amino acid polypeptide with a calculated molecular weight of 18,942 daltons. A 25 amino acid sequence, which was identical to the experimentally determined N-terminal sequence of purified bovine HBGF-8, began 32 amino acids downstream of the proposed initiation codon. It also shows a hydrophobic N-terminal region of 32 amino acid residues which is believed to be a leader sequence. Comparison of predicted amino acid sequences of human and bovine HBGF-8 showed that HBGF-8 is highly conserved. Of 168 amino acid residues, 163 are identical.

Standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, deoxyadenosine-5'-triphosphate (dATP). As is conventional for convenience in the structural representation of a DNA nucleotide sequence, only one strand is usually shown in which A on one strand connotes T on its complement and G connotes C.

Amino acids are shown herein by standard three letter abbreviations as follows:

Abbreviated Designation	Amino Acid
Ala	Alanine
Cys	Cysteine
Asp	Aspartic acid
Glu	Glutamic acid
Phe	Phenylalanine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Lys	Lysine
Leu	Leucine

(continued)

Abbreviated Designation	Amino Acid
Met	Methionine
Asn	Asparagine
Pro	Proline
Gln	Glutamine
Arg	Arginine
Ser	Serine
Thr	Threonine
Val	Valine
Trp	Tryptophan
Tyr	Tyrosine

Claims

1. A human placenta derived heparin-binding growth factor of 168 amino acids having the following amino acid sequence:

```

Met Gln Ala Gln Gln Tyr Gln Gln Gln Arg Arg Lys Phe Ala Ala   15
Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala   30
Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser   45
Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly   60
Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu   75
Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn   90
Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala  105
Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly  120
Ser Leu Lys Arg Ala Leu His Asn Ala Glu Cys Gln Lys Thr Val  135
Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Pro Lys Pro Gln  150
Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys  165
Met Leu Asp                                                    168

```

2. A DNA sequence consisting of a sequence encoding human heparin binding growth factor of 168 amino acids having the following amino acid sequence:

Met Gln Ala Gln Gln Tyr Gln Gln Gln Arg Arg Lys Phe Ala Ala 15
 5 Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala 30
 Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser 45
 Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly 60
 10 Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu 75
 Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn 90
 15 Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala 105
 Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly 120
 Ser Leu Lys Arg Ala Leu His Asn Ala Glu Cys Gln Lys Thr Val 135
 20 Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Pro Lys Pro Gln 150
 Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys 165
 Met Leu Asp 168
 25

3. The cDNA of human heparin-binding growth factor having the following nucleotide sequence:

30

35

40

45

50

55

	GTCAAAGGCA	GGATCAGGTT	CCCCGCCTTC	CAGTCCAAAA	ATCCCGCCAA	50
	GAGAGCCCCA	GAGCAGAGGA	AAATCCAAAG	TGGAGAGAGG	GGAAGAAAGA	100
5	GACCAAGTGAG	TCATCCGTCC	AGAAGGCGGG	GAGAGCAGCA	GCGGCCCAAG	150
	CAGGAGCTGC	AGCGAGCCGG	GTACCTGGAC	TCAGCGGTAG	CAACCTCGCC	200
10	CCTTGCAACA	AAGGCAGACT	GAGCGCCAGA	GAGGACGTTT	CCAACTCAAA	250
	AATGCAGGCT	CAACAGTACC	AGCAGCAGCG	TCGAAAATTT	GCAGCTGCCT	300
	TCTTGGCATT	CATTTTCATA	CTGGCAGCTG	TGGATACTGC	TGAAGCAGGG	350
15	AAGAAAGAGA	AACCAGAAAA	AAAAGTGAAG	AAGTCTGACT	GTGGAGAATG	400
	GCAGTGGAGT	GTGTGTGTGC	CCACCAGTGG	AGACTGTGGG	CTGGGCACAC	450
20	GGGAGGGCAC	TCGGACTGGA	GCTGAGTCCA	AGCAAACCAT	GAAGACCCAG	500
	AGATGTAAGA	TCCCCTGCAA	CTGGAAGAAG	CAATTTGGCG	CGGAGTGCAA	550
	ATACCAGTTC	CAGGCCTGGG	GAGAATGTGA	CCTGAACACA	GCCCTGAAGA	600
25	CCAGAACTGG	AAGTCTGAAG	CGAGCCCTGC	ACAATGCCGA	ATGCCAGAAG	650
	ACTGTCACCA	CTCCCAAGCC	CTGTGGCAAA	CTGACCAAGC	CCAAACCTCA	700
	AGCAGAATCT	AAGAAGAAGA	AAAAGGAAGG	CAAGAAACAG	GAGAAGATGC	750
30	TGGATTAAAA	GATGTCACCT	GTGGAACATA	AAAAGGACAT	CAGCAAACAG	800
	GATCAGTTAA	CTATTGCATT	TATATGTACC	GTAGGCTTTG	TATTCAAAAA	850
35	TTATCTATAG	CTAAGTACAC	AATAAGCAAA	AACAAAAAGA	AAAAAAAAAA	900
	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	950
40	AAAAAAAAAA	A				961

Patentansprüche

1. Aus einer menschlichen Plazenta abgeleiteter Heparin-bindender Wachstumsfaktor aus 168 Aminosäuren mit der nachfolgenden Aminosäuresequenz:

	Met Gln Ala Gln Gln Tyr Gln Gln Gln Arg Arg Lys Phe Ala Ala	15
5	Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala	30
	Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser	45
	Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly	60
10	Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu	75
	Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn	90
	Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala	105
15	Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly	120
	Ser Leu Lys Arg Ala Leu His Asn Ala Glu Cys Gln Lys Thr Val	135
	Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Pro Lys Pro Gln	150
20	Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys	165
	Met Leu Asp	168

- 25 2. DNA-Sequenz,
bestehend aus einer Sequenz, die einen menschlichen Heparin-bindenden Wachstumsfaktor aus 168 Aminosäuren mit der nachfolgenden Aminosäuresequenz kodiert:

30	Met Gln Ala Gln Gln Tyr Gln Gln Gln Arg Arg Lys Phe Ala Ala	15
	Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala	30
	Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser	45
35	Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly	60
	Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu	75
	Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn	90
40	Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala	105
	Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly	120
	Ser Leu Lys Arg Ala Leu His Asn Ala Glu Cys Gln Lys Thr Val	135
45	Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Pro Lys Pro Gln	150
	Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys	165
50	Met Leu Asp	168

3. cDNA von menschlichem Heparin-bindendem Wachstumsfaktor mit der nachfolgenden Nukleotidsequenz:

GTCAAAGGCA GGATCAGGTT CCCCGCCTTC CAGTCCAAAA ATCCCGCCAA 50
 5 GAGAGCCCCA GAGCAGAGGA AAATCCAAAG TGGAGAGAGG GGAAGAAAGA 100
 GACCAGTGAG TCATCCGTCC AGAAGGCGGG GAGAGCAGCA GCGGCCCAAG 150
 CAGGAGCTGC AGCGAGCCGG GTACCTGGAC TCAGCGGTAG CAACCTCGCC 200
 10 CCTTGCAACA AAGGCAGACT GAGCGCCAGA GAGGACGTTT CCAACTCAAA 250
 AATGCAGGCT CAACAGTACC AGCAGCAGCG TCGAAAATTT GCAGCTGCCT 300
 TCTTGGCATT CATTTTCATA CTGGCAGCTG TGGATACTGC TGAAGCAGGG 350
 15 AAGAAAGAGA AACCAGAAAA AAAAGTGAAG AAGTCTGACT GTGGAGAATG 400
 GCAGTGGAGT GTGTGTGTGC CCACCAGTGG AGACTGTGGG CTGGGCACAC 450
 GGGAGGGCAC TCGGACTGGA GCTGAGTCCA AGCAAACCAT GAAGACCCAG 500
 20 AGATGTAAGA TCCCCTGCAA CTGGAAGAAG CAATTGGCG CGGAGTGCAA 550
 ATACCAGTTC CAGGCCTGGG GAGAATGTGA CCTGAACACA GCCCTGAAGA 600
 25 CCAGAACTGG AAGTCTGAAG CGAGCCCTGC ACAATGCCGA ATGCCAGAAG 650
 ACTGTCACCA CTCCCAAGCC CTGTGGCAAA CTGACCAAGC CCAAACCTCA 700
 AGCAGAATCT AAGAAGAAGA AAAAGGAAGG CAAGAAACAG GAGAAGATGC 750
 30 TGGATTAAAA GATGTCACCT GTGGAACATA AAAAGGACAT CAGCAAACAG 800
 GATCAGTTAA CTATTGCATT TATATGTACC GTAGGCTTTG TATTCAAAAA 850
 TTATCTATAG CTAAGTACAC AATAAGCAAA AACAAAAAGA AAAAAAAAAA 900
 35 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 950
 AAAAAAAAAA A 961

40

Revendications

1. Facteur de croissance fixant l'héparine, dérivé du placenta humain, constitué de 168 amino-acides, avant la sé-
 quence d'amino-acides suivante :

45

50

55

Met Gln Ala Gln Gln Tyr Gln Gln Gln Arg Arg Lys Phe Ala Ala 15
 Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala 30
 5 Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser 45
 Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly 60
 Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu 75
 10 Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn 90
 Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala 105
 Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly 120
 15 Ser Leu Lys Arg Ala Leu His Asn Ala Glu Cys Gln Lys Thr Val 135
 Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Pro Lys Pro Gln 150
 Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys 165
 20 Met Leu Asp 168

2. Séquence d'ADN consistant en une séquence codant pour le facteur de croissance fixant l'héparine, d'origine humaine, constitué de 168 amino-acides, ayant la séquence d'amino-acides suivante :

Met Gln Ala Gln Gln Tyr Gln Gln Gln Arg Arg Lys Phe Ala Ala 15
 Ala Phe Leu Ala Phe Ile Phe Ile Leu Ala Ala Val Asp Thr Ala 30
 30 Glu Ala Gly Lys Lys Glu Lys Pro Glu Lys Lys Val Lys Lys Ser 45
 Asp Cys Gly Glu Trp Gln Trp Ser Val Cys Val Pro Thr Ser Gly 60
 Asp Cys Gly Leu Gly Thr Arg Glu Gly Thr Arg Thr Gly Ala Glu 75
 35 Cys Lys Gln Thr Met Lys Thr Gln Arg Cys Lys Ile Pro Cys Asn 90
 Trp Lys Lys Gln Phe Gly Ala Glu Cys Lys Tyr Gln Phe Gln Ala 105
 40 Trp Gly Glu Cys Asp Leu Asn Thr Ala Leu Lys Thr Arg Thr Gly 120
 Ser Leu Lys Arg Ala Leu His Asn Ala Glu Cys Gln Lys Thr Val 135
 Thr Ile Ser Lys Pro Cys Gly Lys Leu Thr Lys Pro Lys Pro Gln 150
 45 Ala Glu Ser Lys Lys Lys Lys Lys Glu Gly Lys Lys Gln Glu Lys 165
 Met Leu Asp 168

3. ADNc d'un facteur de croissance humain fixant l'héparine ayant la séquence de nucléotides suivante :

	GTCAAAGGCA GGATCAGGTT CCCC GCCTTC CAGTCCAAAA ATCCCGCCAA	50
5	GAGAGCCCCA GAGCAGAGGA AAATCCAAAG TGGAGAGAGG GGAAGAAAGA	100
	GACCAGTGAG TCATCCGTCC AGAAGGCGGG GAGAGCAGCA GCGGCCCAAG	150
	CAGGAGCTGC AGCGAGCCGG GTACCTGGAC TCAGCGGTAG CAACCTCGCC	200
10	CCTTGCAACA AAGGCAGACT GAGCGCCAGA GAGGACGTTT CCAACTCAAA	250
	AATGCAGGCT CAACAGTACC AGCAGCAGCG TCGAAAATTT GCAGCTGCCT	300
	TCTTGGCATT CATTTTCATA CTGGCAGCTG TGGATACTGC TGAAGCAGGG	350
15	AAGAAAGAGA AACCAGAAAA AAAAGTGAAG AAGTCTGACT GTGGAGAATG	400
	GCAGTGGAGT GTGTGTGTGC CCACCAGTGG AGACTGTGGG CTGGGCACAC	450
20	GGGAGGGCAC TCGGACTGGA GCTGAGTCCA AGCAAACCAT GAAGACCCAG	500
	AGATGTAAGA TCCCCTGCAA CTGGAAGAAG CAATTTGGCG CGGAGTGCAA	550
	ATACCAGTTC CAGGCCTGGG GAGAATGTGA CCTGAACACA GCCCTGAAGA	600
25	CCAGAACTGG AAGTCTGAAG CGAGCCCTGC ACAATGCCGA ATGCCAGAAG	650
	ACTGTCACCA CTCCCAAGCC CTGTGGCAAA CTGACCAAGC CCAAACCTCA	700
30	AGCAGAATCT AAGAAGAAGA AAAAGGAAGG CAAGAAACAG GAGAAGATGC	750
	TGGATTAAAA GATGTCACCT GTGGAACATA AAAAGGACAT CAGCAAACAG	800
	GATCAGTTAA CTATTGCATT TATATGTACC GTAGGCTTTG TATTCAAAAA	850
35	TTATCTATAG CTAAGTACAC AATAAGCAAA AACAAAAAGA AAAAAAAAAA	900
	AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	950
40	AAAAAAAAAA A	961
45		
50		
55		

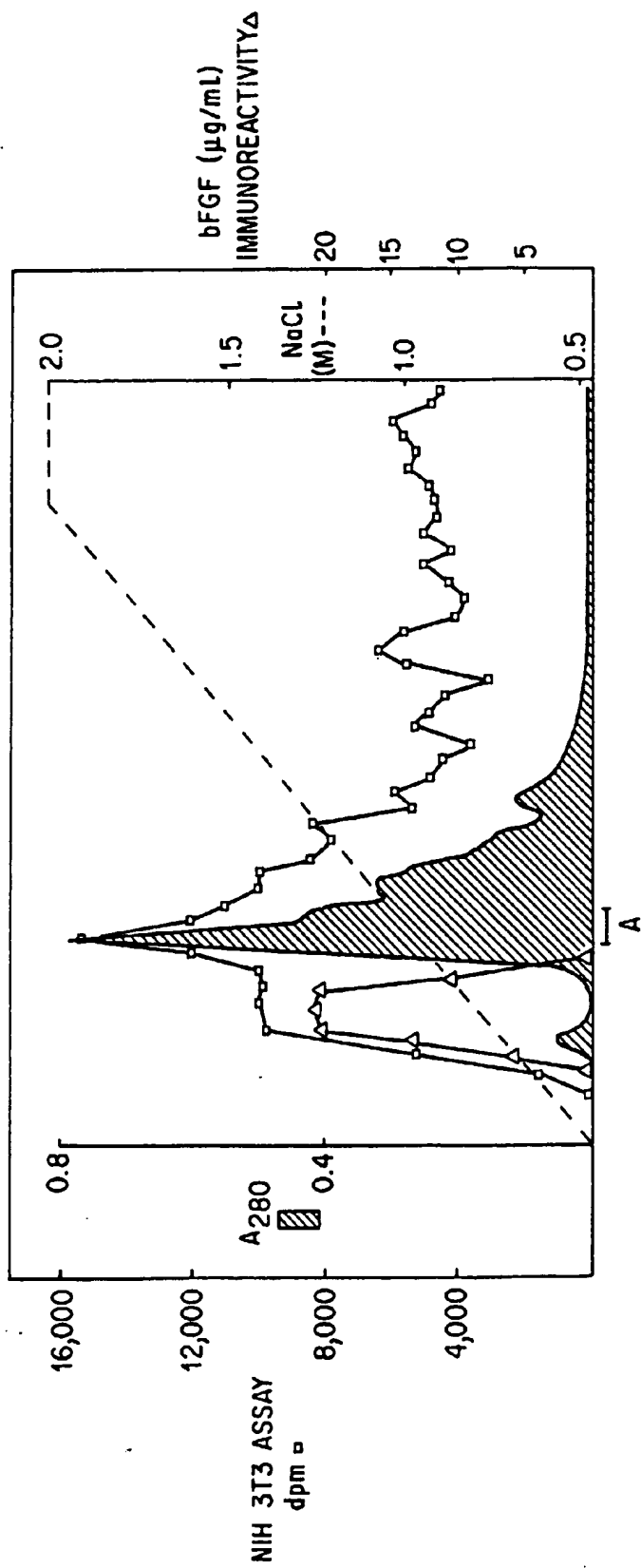


FIG. 1

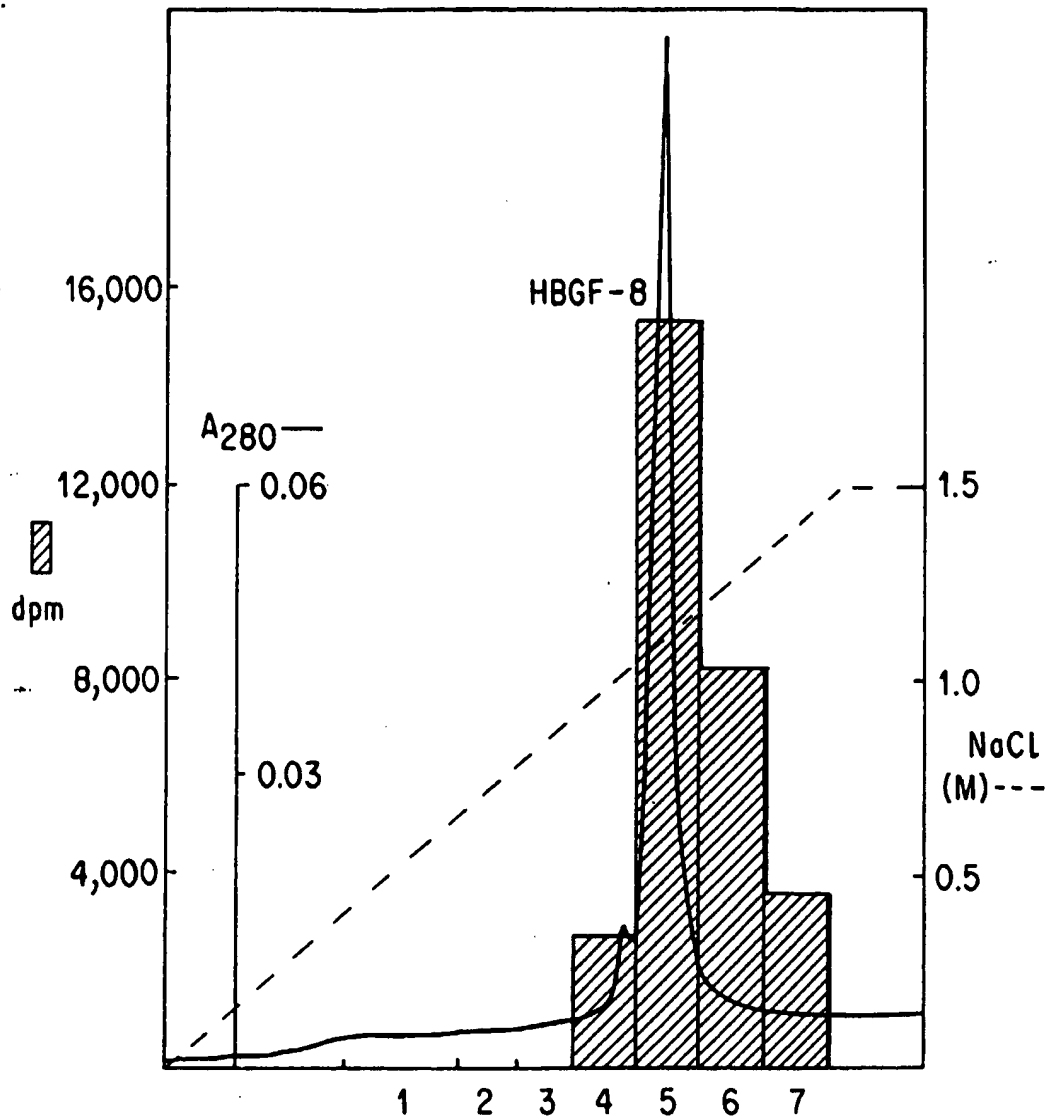


FIG. 2

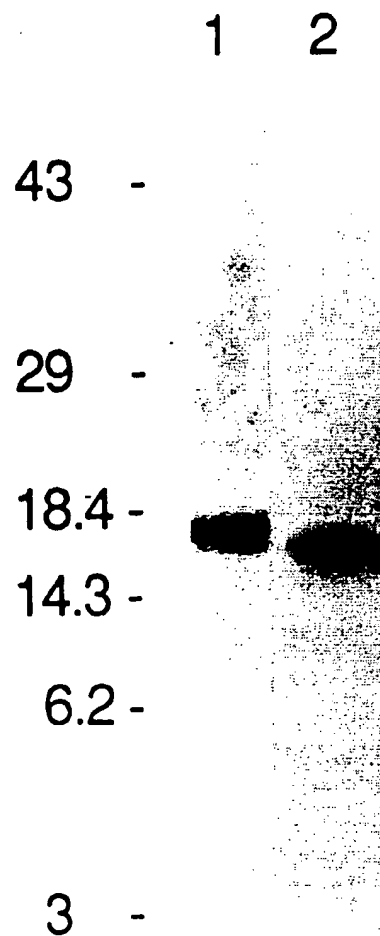


FIG. 3